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MT1-MMP CONTROLS TUMOR-INDUCED ANGIOGENESIS THROUGH THE RELEASE OF SEMAPHORIN 4D*

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Running Title: MT1-MMP regulates Plexin-B1 ligand availability

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The semaphorins are a family of proteins originally identified as regulators of axon growth that recently have been implicated in blood vessel development. The plexins are high-affinity receptors for the semaphorins and are responsible for initiation of signaling upon ligation. Emerging evidence indicates that many human cancers overexpress Semaphorin 4D, which promotes neovascularization upon stimulating its receptor, Plexin-B1, on endothelial cells. However, in order to exert its pro-angiogenic functions, Semaphorin 4D must be processed and released from its membrane bound form to act in a paracrine manner on endothelial cells. Here we show that Semaphorin 4D is a novel target for the membrane-tethered collagenase Membrane Type 1- Matrix Metalloproteinase. We demonstrate that this metalloproteinase, which is not expressed in normal or immortal but non-tumorigenic epithelial cell lines, was present in several head and neck squamous cell carcinoma cell lines and was required for processing and release of Semaphorin 4D into its soluble form from these cells, thereby inducing endothelial cell chemotaxis *in vitro* and blood vessel growth *in vivo*. These results suggest that the proteolytic cleavage of Semaphorin 4D may provide a novel molecular mechanism by which Membrane Type 1-Matrix Metalloproteinase controls tumor-induced angiogenesis.

The semaphorins represent a large family of phylogenetically conserved molecules, both membrane bound and secreted, originally identified by their ability to provide attractive and repulsive axon guidance cues during axon growth (1). Semaphorins have been grouped into eight classes based upon their species of origin and sequence similarity: Classes 1 and 2 are found in invertebrates, Classes 3 to 7 in vertebrates, and Class V is encoded by some non-neurotropic DNA viruses (2). The plexins and the neuropilins are the two main families of receptors for the semaphorins, and they can homodimerize or heterodimerize upon ligand binding depending on whether the semaphorin is soluble or membrane bound (3,4). Neuropilins and their homologues have been shown to complex with Plexin-B1 (5) and Plexin-D1 (6) and, in certain conditions, serve as co-receptors with A family plexins for class 3 semaphorins (4,7). It is the plexins, however, that initiate the signaling cascades upon binding of a semaphorin ligand. There have been nine plexins identified in humans so far, most of which have been shown to regulate neuronal cell growth and contact and nerve fasciculation (8-10). They are grouped into 4 families, A through D, based upon sequence homology. While sharing homology in their extracellular region with the scatter factor receptors such as RON and c-Met, the intracellular region of the plexins contain a unique domain called the Sex-Plex domain that is highly conserved within and across species.

The semaphorins and plexins recently have been implicated in a host of responses including regulation of cell migration (11), immune responses (12), tumor progression (13) and tissue organization during development (14-16). In addition, the functions of proteins involved in the transmission of axonal guidance cues have been expanded to include regulation of blood vessel growth and endothelial precursor cell homing during vessel development (17,18). We and others have observed that Plexin-B1 is highly expressed in endothelial cells and promotes migration and tubulogenesis when bound by its ligand, Semaphorin 4D (Sema4D)[1] (19,20). Surprisingly, while exploring the nature of the molecules expressed in head and neck squamous cell carcinomas (HNSCC), we have recently

observed that Sema4D is highly expressed in HNSCC as well as in some of the most prevalent solid tumors, including breast, prostate, and colon carcinomas (21). These findings suggest that the class IV semaphorins may regulate angiogenesis *in vivo* and raise the possibility that Sema4D could play a role in tumor-induced angiogenesis. However, in order to exert its pro-angiogenic functions, Sema4D, a membrane bound protein, must be processed and released into a soluble form to act in a paracrine manner on endothelial cells.

Semaphorin 4D is known to be expressed on the surface of cells as a homodimer, but it has also been shown to be shed into the surrounding environment through proteolytic cleavage, the mechanism for which has only recently been investigated (22). The protease responsible for Semaphorin 4D cleavage is likely a matrix metalloproteinase (MMP), a group of zinc-dependent enzymes that hydrolyze numerous components of the extracellular matrix, because shedding could be partly inhibited by the MMP inhibitors EDTA and EGTA (22). Upregulation of MMPs in cancer cells has been linked to acquisition of an invasive phenotype, with cells acquiring the ability to digest extracellular matrix substrates and invade underlying tissue and metastasize (23). Indeed, we have found HNSCC secrete a soluble form of Sema4D that acts through Plexin-B1 on the surface of endothelial cells to enhance tumor growth and survival by promoting angiogenesis (21).

In this study, we use general and specific inhibitors of MMPs and knockout MEFs to demonstrate that Sema4D is a novel target for membrane type 1-MMP (MT1-MMP, also called MMP14), a member of a family of metalloproteinases that are tethered to the cell membrane and confer peri-cellular proteolytic activity but also participate in the processing of membrane bound receptors and proteins (24). We found that MT1-MMP, while not expressed in non-tumorigenic epithelial cell lines, was present in several head and neck squamous cell carcinoma cell lines. MT1-MMP was required for processing and release of Sema4D into its soluble form from these cells, thereby inducing endothelial cell chemotaxis *in vitro* and tumor-induced angiogenesis *in vivo*. These results suggest that the proteolytic cleavage of Sema4D may provide a novel molecular mechanism by which MT1-MMP controls tumor-induced angiogenesis.

Experimental Procedures

Cell Culture - HNSCC cell lines and MT1-MMP and MMP-2 wild-type and knockout MEFs were grown in DMEM (Sigma, St. Louis, MO). The human T cell line, Jurkat, was grown in RPMI (Sigma). Porcine aorta endothelial cells were cultured in HAM F-12 media (Sigma). All media were supplemented with 10% fetal bovine serum and 100 units/ml penicillin/ streptomycin/ amphotericin B (Sigma). Cells were treated with GM6001 (Chemicon, Temecula, CA), TIMP-1 (Sigma), or TIMP-2 (Sigma) where indicated.

Immunoblot Analysis - Analysis of whole cell extracts was performed as previously described (25). For transmembrane proteins, cells were processed as previously described (26), with minor modifications. Briefly, cells were lysed in buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.5% pre-condensed Triton X-114) supplemented with protease and phosphatase inhibitors for 15 min at 4°C, and centrifuged at 16,000 x g for 15 min at 4°C. The supernatant was removed and incubated at 37°C for 2 min, after which it separated into an upper (aqueous) layer, which was discarded, and a lower (detergent) phase that was quantified and loaded onto the gel. For analysis of conditioned media, 4.5 ml of serum free media was placed on cells growing in 10 cm dishes and left overnight to concentrate molecules released to the media. Sample buffer was added directly to the media after collection and 50 μl loaded for immunoblotting. Following gel electrophoresis, a transfer was done onto a PVDF membrane (Immobilon P, Millipore, Bedford, MA), which was incubated with the appropriate antibodies: Semaphorin 4D (BD Transduction Labs, BD Biosciences, Palo Alto); Tubulin (Pharmingen, BD Biosciences); EGFR (SC03, Santa Cruz, California); MT1-MMP (Chemicon). Proteins were detected using the SuperSignal chemiluminescence system (Pierce, Rockford, IL).

Gelatin Zymography - Gelatin zymography was performed as previously described (27). Briefly, SDS PAGE gelatin gels (Invitrogen, Carlsbad, CA) were loaded with 25 μg of protein and run as

a standard immunoblot (see above). The gels were soaked in renaturing buffer for 30 min., incubated overnight in developing buffer, and stained with Coomassie Blue.

Migration Assays - Serum free media containing the indicated cell type or chemoattractant was placed in the bottom well of a Boyden chamber while serum free media containing endothelial cells was added to the top. The two chambers were separated by a PVPF membrane (Osmonics, GE Water Technologies, Trevose, PA, 8 μ pore size) coated with 10 μg/ml fibronectin (Invitrogen). After 7 h, the chamber was disassembled and the membrane stained with Diff-Quick Stain (Diff-Quick, Dade Behring, Deerfield, Illinois), placed on a glass slide and scanned. Densitometric quantitation was performed with NIH image software.

Lentivirus Infections - The shRNA sequences for human MT1-MMP were obtained from Cold Spring Harbor Laboratory's RNAi library (RNAi Codex, (http://katahdin.cshl.org:9331/homepage/portal/scripts/main2.pl) (28,29). Oligonucleotides based upon the following sequence, identified as shRNA2, worked best to knock down MT1-MMP:
5'TGCTGTTGACAGTGAGCGCAGCCTTCCA
ACTCTGGAGTAATAGT
GAAGCCACAGATGTATTACTCCAGAGTTG
GAAGGCTTTGCCTACTGCCTCGGA3'.
shRNA1, used as a control, was generated by the following oligos:
5'TGCTGTTGACAGTGAGCGCTGGGCTGAA
AGTGACTGGGAATAGTGAAGCCACAGATG
TATTCCCAGTCACTTTCAGCCCAATGCCTA
CTGCCTCGGA3'. Oligos were digested with XhoI/EcoRI, and cloned into pSHAG MAGIC2 (29), an entry vector for the Gateway cloning system (Invitrogen). In the case of wild-type MT1-MMP, the sequence was cloned into pSHAG MAGIC2 at SalI/XhoI. For catalytically inactive MT1-MMP, the E240A mutation was generated using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) prior to cloning into pSHAG MAGIC2. An LR reaction was performed to transfer the inserts into pWPI GW, a Gateway compatible CSCG based retroviral destination vector. Virus was prepared as previously reported (30) using 293T cells as the packaging cells. Target cells were infected with viral supernatants for 24 h at 37°C in the presence of 8 μg/ml of polybrene (hexadimethrine bromide, Sigma).

Transfection - Where indicated, cells were transfected prior to immunoblotting, migration assays or angiogenesis assays with pcDNA 3.1/ GS/ Sema4D or pSecTag2B Sema4D, or the appropriate control vectors, using Lipofectamine Plus (Invitrogen), supplemented with CombiMag transfection agent (Oz Biosciences, Marseille, France), in order to increase transfection efficiency.

In Vivo Cultrex Assay - A DIVAA assay (Trevigen, Gaithersburg, MD) was performed as previously described (31), with modifications. Briefly, angioreactors were filled with 18 μl of Cultrex reconstituted basement membrane substrate (Trevigen) containing 37.5 ng VEGF and 12.5 ng bFGF (positive control), PBS (negative control) or 1 x 10^6 HN12 cells in serum free DMEM, treated as indicated prior to the assay. These were implanted subcutaneously into 6-week old nude mice (Jackson Laboratory, Bar Harbor, ME). Nine days after implantation, the mice were sacrificed and the angioreactors removed, photographed and processed with FITC-labeled Griffonia lectin (FITC-lectin), an endothelial cell selective reagent (32,33), to quantify invasion of endothelial cells into the angioreactors. Fluorescence was determined in a plate reader as mean relative fluorescence units for triplicate assays.

RESULTS

We previously have shown that HNSCC cells secrete a soluble form of Sema4D that acts through Plexin-B1 on the surface of endothelial cells to enhance angiogenesis in mouse tumor xenografts (21). Therefore, in search of the proteolytic activity responsible for Sema4D release, we used a representative HNSCC cell line, HN12, which exhibits Sema4D-mediated angiogenesis and focused our attention on metalloproteinases based upon their potential role in Sema4D proteolysis in T-lymphocytes (22). HN12 cells failed to release soluble Sema4D into the conditioned medium when treated with GM6001, a general metalloproteinase inhibitor (34) (Fig. 1*A*), showing that a metalloproteinase activity was required for Sema4D release. Tissue inhibitor of metalloproteinases (TIMP)-1, which

inhibits soluble matrix metalloproteinases was unable to block Sema4D release (Fig. 1*B*). In contrast, TIMP-2, which inhibits both soluble and membrane-type MMPs, completely inhibited Sema4D release (Fig. 1*C*). This pattern of proteinase inhibitor sensitivity, where Sema4D release is inhibited by TIMP-2 but not TIMP-1, suggested that Sema4D release could be mediated by the MT-MMPs.

Of the six human MT-MMPs, MT1-MMP is most frequently overexpressed in tumors and associated with tumor progression and angiogenesis (35-37). To address the possible role of MT1-MMP in Sema4D release, mouse embryonic fibroblasts (MEFs) from MT1-MMP knockout mice (38) and wild-type littermates were transfected with a full length, membrane bound form of Sema4D. Equal levels of Sema4D were seen in total cell lysates from wild-type and knockout MEFs transfected with the Sema4D construct (Fig. 2*A*). However, MT1-MMP knockout cells failed to release Sema4D into conditioned medium (Fig. 2*A*). MT1-MMP knockout MEFs transfected with a Sema4D plasmid engineered to produce a secreted form of Sema4D (39) released Sema4D into conditioned medium (Fig. 2*B*), indicating that the cellular machinery involved in the processing and secretion of soluble proteins remained intact.

Although zymograms still showed significant secretion of pro-MMP-2 (gelatinase A) in MT1-MMP knock-out MEFs as well as the wild-type cells (Fig. 2*A*, *lower right, lower band*), MT1-MMP is known to play an important role in MMP-2 activation (40). Thus, we challenged whether our results were due to a decrease of pro-MMP-2 activation by MT1-MMP by transfecting Sema4D into MMP-2 knockout MEFs. Both wild-type and MMP-2 knockout MEFs exhibited equal levels of Sema4D in cell lysates and released equal levels of Sema4D in the conditioned media, establishing that MMP-2 activity is not necessary for Sema4D release (Fig. 2*C*).

To determine if the catalytic activity of MT1-MMP was required for Sema4D release, we generated lentiviruses expressing either wild-type MT1-MMP or a catalytically inactive MT1-MMP containing an active site point mutation (E240A) (41) and infected wild-type and MT1-MMP knockout MEFs transfected with full-length Sema4D. Re-expression of MT1-MMP in knockout MEFs by infection with lentiviruses coding for wild-type MT1-MMP and its E240A mutant was confirmed by western blot analysis of membrane extracts (Fig. 3*A*, lane *3* and *4*, respectively). Immunoblots for Sema4D demonstrated its expression in cells transfected with the full length Sema4D (Fig. 3*B*, *lysate, upper panel*, lanes *4*, *5*, *8* and *9*) and in media conditioned by control infected wild-type MEFs (Fig. 3*B*, lane *4*, *CM, upper panel*) but in MT1-MMP knockout MEFs only when infected with lentivirus coding for wild-type MT1-MMP (Fig. 3*B*, lane *8*, *CM, upper panel*) or when transfected with pSecTag2B Sema4D, which codes for a secretory form of Sema4D (Fig. 3*B*, lane *11*, *CM, upper panel*). Secretion of soluble Sema4D into the conditioned media could not be rescued in MT1-MMP knockout MEFs infected with the catalytically inactive MT1-MMP E240A mutant (lane *9*, *CM, upper panel*). In addition, wild-type MEFs infected with the E240A mutant secreted much lower levels of Sema4D (Fig. 3*B*, lane *5*, *CM, upper panel*), suggesting that the inactive mutant may compete with the endogenous enzyme. Zymograms (Fig. 3*B*, *CM, lower panel*) supported these observations, as MMP-2 activity was decreased in all cells that lack functional MT1-MMP (lanes *3*, *7*, *9*, *10*, and *11*) or where the MT1-MMP E240A mutant is exerting a potential dominant negative effect over the endogenous enzyme (lanes *3* and *5*).

To determine the biological significance of MT1-MMP-induced Sema4D release, we used full length Sema4D transfected wild-type and MT1-MMP knockout MEFs as the chemoattractants for endothelial cells in *in vitro* migration assays. While both wild-type and knockout MEF controls exhibited some chemotactic effects upon endothelial cells (Fig. 3*C*, *MT1-MMP: wt, KO*, respectively), wells containing wild-type MEFs transfected with Sema4D (Fig. 3*C*, *wt, S4D*) induced a robust endothelial cell migration, while the MT1-MMP knockouts cells transfected with Sema4D failed to do so (Fig. 3*C*, *KO, S4D*). We next performed an *in vivo* angiogenesis assays in nude mice. Sema4D-transfected wild-type and MT1-MMP knockout MEFs infected with lentiviruses transducing either the wild-type MT1-MMP or the E240A mutant were mixed with reconstituted basement membrane material and placed in an

open-ended silicone tube angioreactor, which was implanted subcutaneously into nude mice (31). After nine days, reactors containing wild-type and knockout MEFs infected with control lentivirus demonstrated an angiogenic response at levels only slightly higher than that seen for negative controls (Fig. 3*D*, lanes *4* and *6*), whereas MT1-MMP wild-type MEFs expressing wild-type Sema4D were able to induce blood vessel growth comparable to that seen in VEGF and FGF filled control angioreactors (Fig. 3*D*, lane *7*). In contrast, MT1-MMP knockout MEFs expressing the wild-type form of Sema4D failed to do so (Fig. 3*D*, lane *5*) but reacquired the ability to induce blood vessel ingrowth when infected with lentivirus transducing wild-type MT1-MMP (Fig. 3*D*, lane *10*) but not the E240A catalytic site mutant (Fig. 3*D*, lane *9*). The pro-angiogenic phenotype could also be rescued in knockout MEFs by transfecting the secreted form of Sema4D, indicating that loss of Sema4D release is sufficient to abrogate the pro-angiogenic response in these cells (Fig. 3*D*, *Sec S4D*, lane *8*) and that a catalytically active form of MT1-MMP is necessary for cells to release Sema4D and to promote angiogenesis *in vivo*.

We next determined if tumor cells employ this paracrine MT1-MMP/Sema4D pathway to promote angiogenesis, as Sema4D is highly expressed in HNSCC cell lines compared to normal or immortalized human oral keratinocytes (21). Interestingly, HNSCC cells also overexpress MT1-MMP, whereas the immortal but non-tumorigenic keratinocyte cell lines HaCaT and HeLa do not (Fig. 4*A*). To examine the role of MT1-MMP in Sema4D release from HNSCC cells, we reduced the expression of endogenous MT1-MMP by generating lentiviruses expressing short hairpin RNAs (shRNAs) for MT1-MMP (28). As shown in Fig. 4*B*, we identified an shRNA construct that caused a marked reduction in MT1-MMP protein levels (*shRNA2*, lane *5*) and MMP-2 activation (Fig. 4*C*, *lower panel*, lane *4*), which, when expressed in HN12 cells, markedly reduced Sema4D release into the conditioned medium (Fig. 4*C*, *upper panel*, lane *4*).

To determine the significance of MT1-MMP-mediated Sema4D release for tumor-induced angiogenesis, we used HN12 cells infected with lentiviruses expressing the functional MT1-MMP shRNA (Fig. 4A, *shRNA2*, lane *5*) or a control shRNA (Fig. 4A, *shRNA1*, lane *4*) in *in vivo* angiogenesis assays in nude mice (31). The results showed significant blood vessel growth into angioreactors containing HN12 cells infected with control virus (Figure 4*D*, *upper panel*, *HN12*, *reactor 3*), similar to that seen for positive controls (Fig. 4*D*, *upper panel*, *VEGF/FGF*, *reactor 2*) but only slight growth into reactors containing HN12 cells infected with MT1-MMP shRNA (Fig. 4*D*, *upper panel*, *HN12*, *MT1-MMP shRNA*, *reactor 4*), thus supporting a critical role for MT1-MMP in tumor induced angiogenesis. Importantly, blood vessel growth could be fully restored in reactors containing HN12 cells infected with MT1-MMP shRNA expressing virus when these cells were transfected with a plasmid expressing the soluble form of Sema4D (Fig. 4*D*, *upper panel*, *Sec S4D*, *reactor 5*). Measurement of FITC-lectin binding to the endothelial contents of reactors supported these findings and demonstrated reduced fluorescence in reactors that contained HN12 cells transduced with MT1-MMP shRNA (Fig. 4*D*, *bottom panel*, *HN12*, *MT1-MMP shRNA*, *reactor 4*) compared to the robust fluorescence seen in reactors containing control-infected HN12 cells (Fig. 4D, *right panel*, *HN12*, *reactor 3*). These results support the notion that MT1-MMP may contribute to the ability of carcinoma cells to induce angiogenesis by promoting the release of Sema4D.

DISCUSSION

While genetic and biochemical studies have implicated semaphorins and their receptors, the plexins, in numerous aspects of neural development, evidence now suggests that the plexin-semaphorin signaling system can control blood vessel growth and development as well (18). We have recently observed that Plexin-B1 is highly expressed in endothelial cells and is able to promote endothelial cell migration and tubulogenesis in response to Sema4D (19). Though Sema4D is a membrane bound protein, some semaphorins are known to exist in both membrane-bound and secreted forms (42). We have observed spontaneous processing of membrane bound Sema4D into a smaller form that is released by HNSCC into to the surrounding tissue culture media, findings aligned with recent observations of the presence of Sema4D as a

soluble protein produced by T lymphocytes through cleavage of the membrane form at a cysteine residue located immediately after the transmembrane domain (22). This raises the possibility that Sema4D may act at a distance similar to other proangiogenic molecules that promote endothelial cell chemotaxis, though the mechanism of shedding from the cell surface has remained elusive. Here we show that MT1-MMP is required for this process.

MT1-MMP is a member of a MMP subfamily that consists of six enzymes, MT(1-6)-MMP, all of which share peptide sequence homology (43). Like other MMPs, MT1-MMP is synthesized as a zymogen that requires proteolytic processing to generate its active, membrane-tethered form. Once activated, MT1-MMP has been shown to be crucial for pericellular collagenolysis and subsequently is considered a key enzyme that contributes to tumor cell invasion and metastasis through direct ECM degradation (43). MT1-MMP is also known to act on other proteinases and MMPs, proteinase inhibitors, growth factors and their receptors, chemokines and cytokines, and cell adhesion molecules, thereby influencing cell migration, proliferation, and apoptosis by shedding cell surface molecules or by producing biologically functional fragments from ECM components (37,44). Consistent with its role in the migration and invasion of malignant cells, MT1-MMP is frequently overexpressed in aggressive, metastatic neoplasms, such as observed in cancers of the lung, colon, liver, breast, brain, head and neck, ovary, and uterine cervix (45,46).

In addition to its other numerous pro-tumorigenic effects, we demonstrate that MT1-MMP is necessary to generate soluble Sema4D. We focused on this MMP based upon the loss of Sema4D shedding following the administration of various MMP inhibitors. We show that MT1-MMP-mediated Sema4D shedding can induce endothelial cell chemotaxis *in vitro*, and that HNSCC cell lines express this enzyme while non-invasive epithelial cell lines do not. Expression of MT1-MMP in HNSCC cells appears to be important for tumor-induced angiogenesis, as knocking down MT1-MMP expression with lentiviruses expressing MT1-MMP shRNAs greatly reduced the response in an *in vivo* angiogenesis assay, while re-introduction of MT1-MMP into knockout MEFs restored the angiogenic phenotype.

MT1-MMP expression in some cells is able to induce activation of the extracellular signal-regulated protein kinase (ERK) cascade and gene transcription under the control of the serum response element (SRE) (47). Therefore, we generated a catalytically dead form of MT1-MMP and demonstrated that it is the enzyme's catalytic activity that is, in fact, necessary for processing and shedding of Sema4D. Our catalytically inactive mutant was also able to compete with endogenous protein to reduce, though not completely block, Sema4D cleavage when introduced into wild-type MEFs through lentiviral gene transfer. Taken together, these results strongly suggest that MT1-MMP acts upon Sema4D on the cell surface to cleave and release this protein, which then diffuses out and is able to exert its pro-angiogenic effects at a distance. However, we cannot rule out the possibility that an intermediate protein or proteins exist in this pathway, and it is the activation of the intermediates that then go on to process Sema4D from the cell surface.

Tumor progression and metastasis in large part depend upon the acquisition of an angiogenic capacity. Fast growing tumors become hypoxic because the tumor cells overwhelm the ability of the vasculature to meet their high metabolic demands. Paradoxically, it is the hypoxic environment generated in a tumor as it rapidly outgrows its blood supply that eventually leads to the switch from an avascular to a neovascular phenotype (48), a process that turns on the production of membrane bound pro-angiogenic proteins and soluble survival factors that induce proliferation and migration in surrounding endothelial cells (49). Recent reports have identified MT1-MMP as a hypoxia-induced gene product (50-52). Interestingly, we have found evidence that Sema4D might be upregulated in some HNSCC cell lines upon exposure to hypoxic conditions as well (unpublished observations). These observations raise the possibility of the existence of a pro-angiogenic mechanism whereby tumor cells concomitantly increase expression of MT1-MMP and Sema4D as the neoplasms acquires a neovascular phenotype. Sema4D is cleaved from the surface of the tumor cells in a MT1-MMMP-dependent manner, thereby

facilitating angiogenesis by acting as a chemotactic agent for Plexin-B1 expressing endothelial cells (Fig. 5). These possibilities are under current investigation. Our present findings indicate that the MT1-MMP-dependent proteolytic cleavage of Sema4D may play a critical role in tumor-induced angiogenesis, and therefore may represent new fronts of attack in the anti-angiogenic therapy of cancer.

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FOOTNOTES

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[1]The abbreviations used are: Sema4D, Semaphorin 4D; HNSCC, head and neck squamous cell carcinoma; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-MMP; MEFs, mouse embryonic fibroblasts; TIMP, tissue inhibitor of metalloproteinase; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

FIGURE LEGENDS

Fig. 1. A TIMP-1 insensitive, TIMP-2 sensitive metalloproteinase mediates the release of Sema4D from tumor cells. *A,* Western blot analysis for Sema4D (S4D) (*upper panels)* in HN12 cell lysates and conditioned media (CM), following treatment with vehicle (-) or 1 μM of GM6001 (+), *B*, TIMP-1, and *C*, TIMP-2. Tubulin levels (*lower panels*) indicate equal loading of cell lysates.

Fig. 2. MT1-MMP is necessary for the release of Sema4D *A*, Sema4D (S4D) protein levels in cell lysates and conditioned media (CM) from wild-type (wt) and MT1-MMP knockout (KO) MEFs transfected with a full-length Sema4D. *B*, Sema4D (S4D) protein levels in cell lysates and conditioned media (CM) from wild-type (wt) and MT1-MMP knockout (KO) MEFs transfected with a secreted form (B) of Sema4D. *C*, Sema4D (S4D) levels in lysates and conditioned media (CM) from wild-type (wt) and MMP-2 knockout (KO) MEFs transfected with full-length Sema4D. Tubulin (*lower left panels*) and zymograms exhibiting equal MMP-9 levels (*lower right panels, upper lysis zone*) indicate equal loading of lysates and conditioned media, respectively (*A* and *C*).

Fig. 3. MT1-MMP is necessary for induction of Sema4D-dependent angiogenesis. *A*, Membrane extracts of wild-type (MT1-MMP MEF: wt) and knock-out (KO) MEFs, infected with control lentivirus (C) or lentivirus coding for wild-type or the MT1-MMP E240A mutant. EGFR levels (*lower panel*) indicate equal loading of total and membrane proteins. *B*, Sema4D (S4D) in lysates and media conditioned by wild-type and MT1-MMP knockout MEFs transfected with control vector (DNA: C) or a vector coding for Sema4D (S4D) and infected with control lentivirus or virus coding for MT1-MMP wild-type or E240A mutant (C, wt, and E240A, respectively). Zymograms exhibiting equal MMP-9 levels demonstrate equal loading of conditioned media (*lower panels, upper lysis zone*). *C*, Endothelial cell migration toward control (DNA: C) or Sema4D (S4D) transfected wild-type (MT1-MMP: wt) and knockout (KO) MEFs, expressed as a fold-increase over 0.1% BSA control wells. The bars represent the average ± SEM of six experiments. *D*, *In vivo* angiogenesis assay using MT1-MMP wild-type (MEF: wt) and knockout (KO) MEFs transfected with control vector or a vector coding for full length Sema4D (S4D) or the secreted form of Sema4D (Sec S4D) and infected with control lentivirus or virus coding for the wild-type or the E240A mutant form of MT1-MMP. The bar graph demonstrates the average ± SEM of three experiments.

Fig. 4. Tumors induce angiogenesis by MT1-MMP-mediated Sema4D release. *A*, Immunoblot demonstrating MT1-MMP expression in a panel of HNSCC cell lines but not in the epithelial cell lines HaCaT and HeLa (*upper panel*). Wild-type and knockout MEFs were used as positive and negative controls, respectively. Tubulin was used as a loading control (*lower panel*). *B*, Immunoblot of membrane extracts for MT1-MMP in HN12 cells, infected with control lentiviruses (C) or viruses expressing MT1-MMP shRNAs (shRNA 1 and 2). Tubulin levels (*lower panel*) indicate equal proteins in the corresponding total cell lysates. *C*, Sema4D (S4D) in lysates and conditioned media (CM) from HN12 cells infected with control (C) or MT1-MMP shRNA (shRNA) lentiviruses (*upper panel*). Zymograms exhibiting equal MMP-9 levels demonstrate equal loading of conditioned media (*lower right panel, upper lysis zone*). *D*, HN12 cells infected with control virus (HN12, reactor 3) or virus coding for MT1-MMP shRNA (HN12, MT1-MMP shRNA, reactor 4) or transfected with a plasmid coding for the soluble form of Sema4D (HN12, MT1-MMP shRNA, Sec S4D, reactor 5) were used in an *in vivo* angiogenesis assay. The photo shows blood vessel growth into angioreactors in millimeters. The bar graph demonstrates the average ± SEM of three experiments.

Fig. 5. A model for MT1-MMP-induced paracrine stimulation of Plexin-B1-dependent angiogenesis. MT1-MMP expressed on the surface of tumor cells cleaves membrane bound Sema4D, which can then bind Plexin-B1 on endothelial cells of the tumor stroma initiating an angiogenic response.

Figure 1

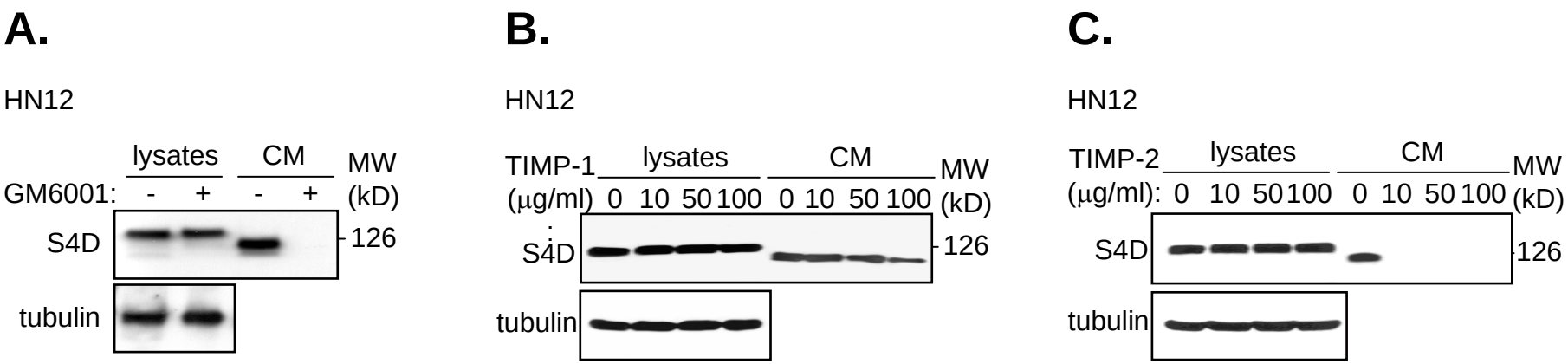

A.
HN12
lysates
CM
MW
GM6001:
-
+
-
+
(kD)
S4D
126
tubulin
B.
HN12
TIMP-1
(μg/ml)
lysates
CM
MW
0
10
50
100
0
10
50
100
(kD)
S4D
126
tubulin
C.
HN12
TIMP-2
(μg/ml):
lysates
CM
MW
0
10
50
100
0
10
50
100
(kD)
S4D
126
tubulin

Figure 2

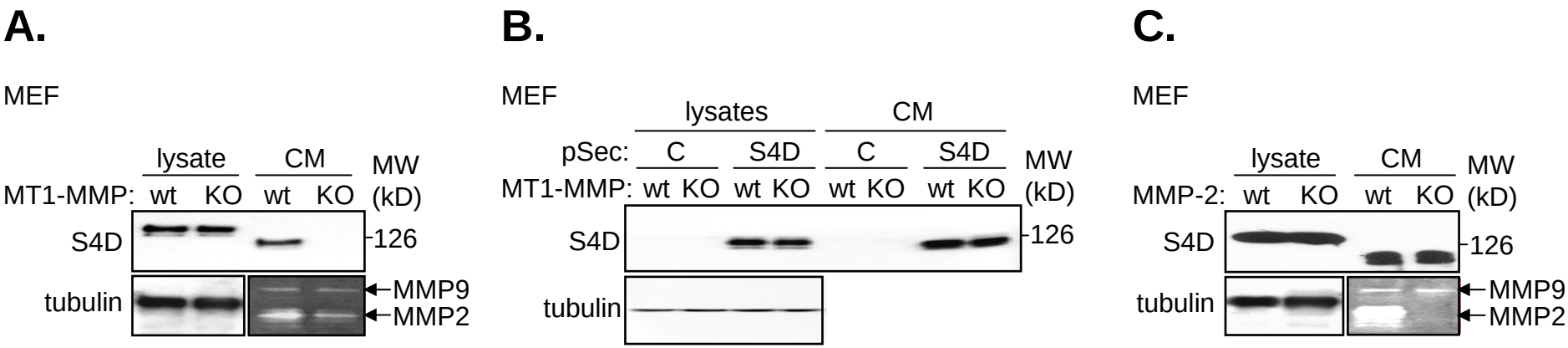

A.
MEF
lysate
CM
MW
MT1-MMP: wt KO wt KO
(kD)
S4D
126
tubulin
MMP9
MMP2
B.
MEF
lysates
CM
pSec: C S4D C S4D
MW
MT1-MMP: wt KO wt KO wt KO wt KO
(kD)
S4D
126
tubulin
C.
MEF
lysate
CM
MW
MMP-2: wt KO wt KO
(kD)
S4D
126
tubulin
MMP9
MMP2

Figure 3

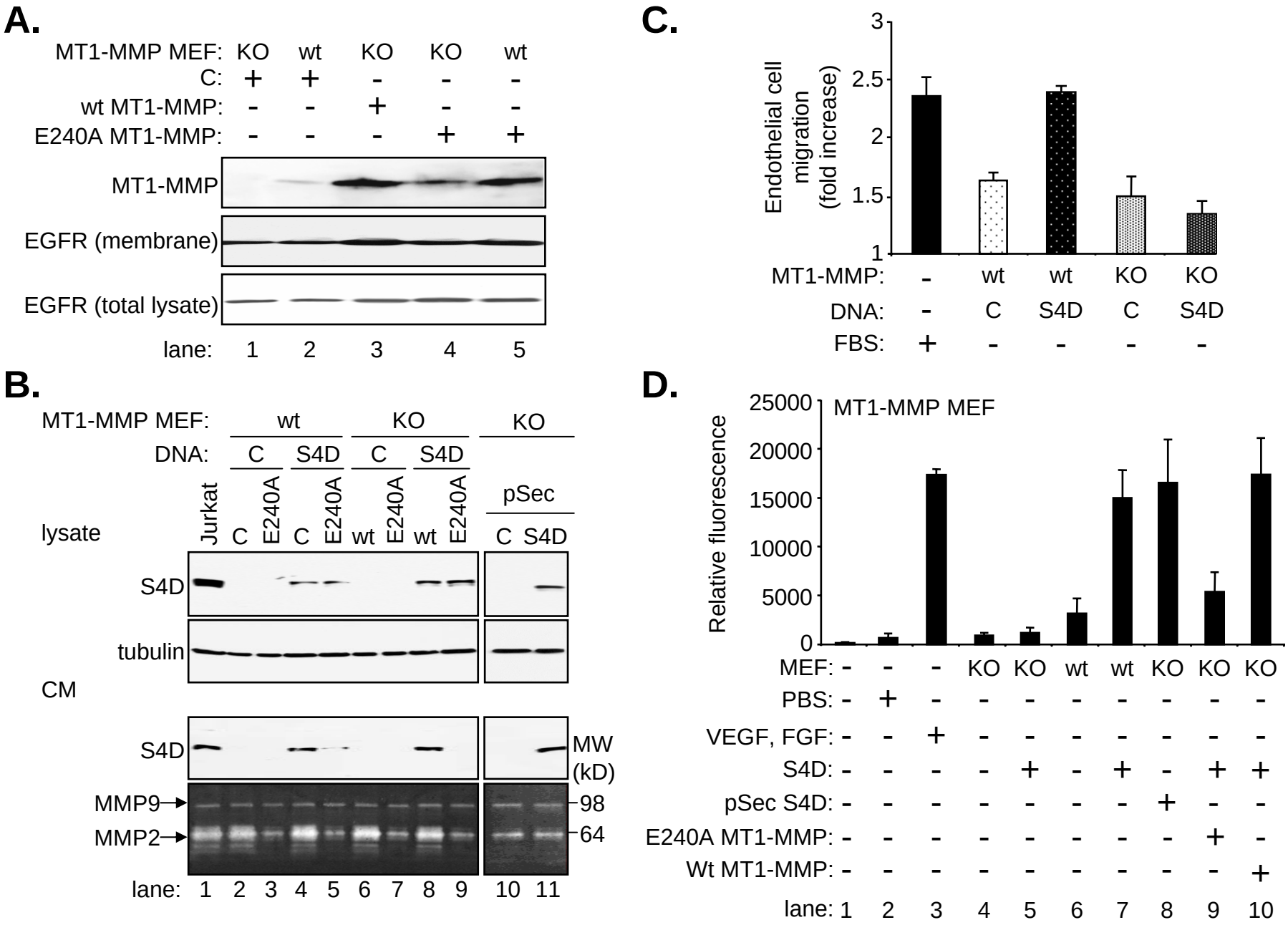

A.
MT1-MMP MEF: KO wt KO KO wt
C: + + - - -
wt MT1-MMP: - - + - -
E240A MT1-MMP: - - - + +
MT1-MMP
EGFR (membrane)
EGFR (total lysate)
lane: 1 2 3 4 5
B.
MT1-MMP MEF: wt KO KO
DNA: C S4D C S4D
Jurkat C E240A C E240A wt E240A wt E240A pSec C S4D
lysate
S4D
tubulin
CM
S4D
MMP9
MMP2
MW (kD)
98
64
lane: 1 2 3 4 5 6 7 8 9 10 11
C.
Endothelial cell migration (fold increase)
3
2.5
2
1.5
1
MT1-MMP: - wt wt KO KO
DNA: - C S4D C S4D
FBS: + - - - -
D.
MT1-MMP MEF
Relative fluorescence
25000
20000
15000
10000
5000
0
MEF: - - - KO KO wt wt KO KO KO
PBS: - + - - - - - - - -
VEGF, FGF: - - + - - - - - - -
S4D: - - - - + - + - + +
pSec S4D: - - - - - - - + - -
E240A MT1-MMP: - - - - - - - - + -
Wt MT1-MMP: - - - - - - - - - +
lane: 1 2 3 4 5 6 7 8 9 10

Figure 4

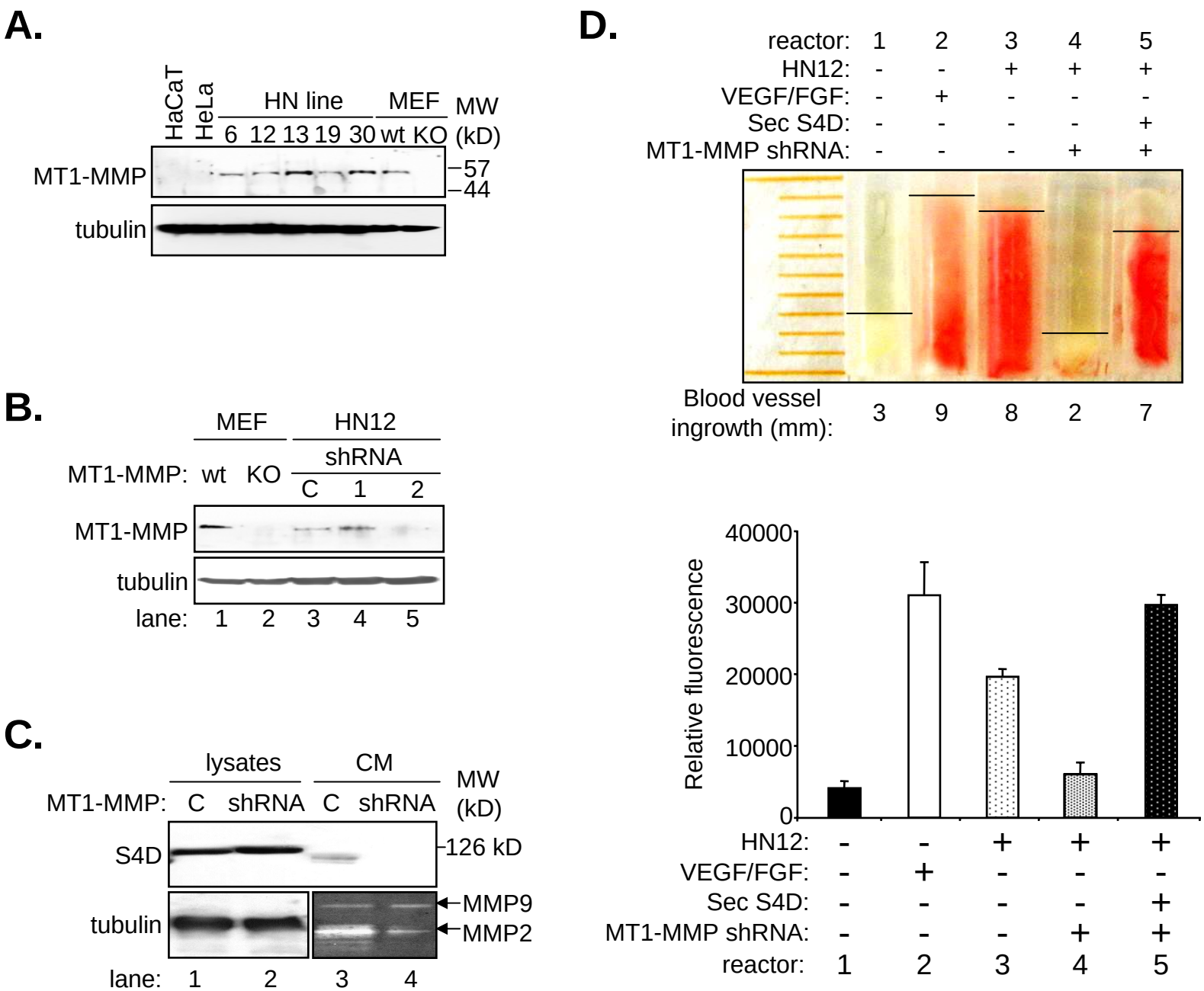
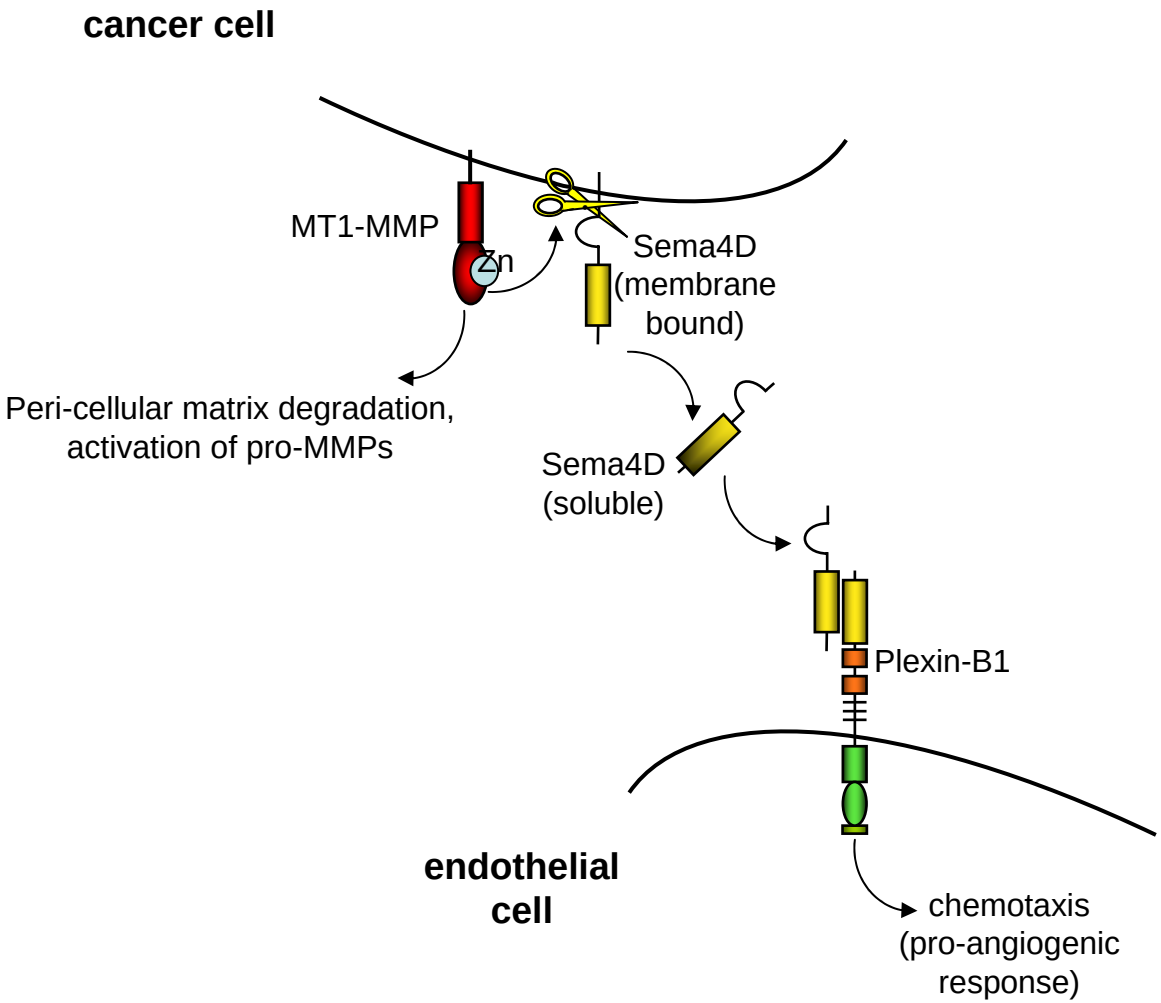

A.
HaCaT
HeLa
HN line
MEF
6 12 13 19 30 wt KO
MW (kD)
MT1-MMP
57
44
tubulin
B.
MEF
HN12
shRNA
MT1-MMP: wt KO C 1 2
MT1-MMP
tubulin
lane: 1 2 3 4 5
C.
lysates
CM
MT1-MMP: C shRNA C shRNA
MW (kD)
S4D
126 kD
tubulin
MMP9
MMP2
lane: 1 2 3 4
D.
reactor: 1 2 3 4 5
HN12: - - + + +
VEGF/FGF: - + - - -
Sec S4D: - - - - +
MT1-MMP shRNA: - - - + +
Blood vessel ingrowth (mm): 3 9 8 2 7
Relative fluorescence
40000
30000
20000
10000
0
HN12: - - + + +
VEGF/FGF: - + - - -
Sec S4D: - - - - +
MT1-MMP shRNA: - - - + +
reactor: 1 2 3 4 5

Figure 5


cancer cell
MT1-MMP
Zn
Sema4D
(membrane
bound)
Peri-cellular matrix degradation,
activation of pro-MMPs
Sema4D
(soluble)
Plexin-B1
endothelial
cell
chemotaxis
(pro-angiogenic
response)